



Original article

In vivo identification of novel TGIF2LX target genes in colorectal adenocarcinoma using the cDNA-AFLP method



Gholam Reza Mobini ^{a,b,1}, Arefeh Ghafari ^{c,1}, Saeid Amanpour ^d, Roohollah Fateh ^e, Mohammad Hossein Ghahremani ^{b,f}, Samad Muhammadnejad ^{c,g}, Ahmad Reza Dehpour ^{h,i}, Abolfazl Akbari ^j, Seyed Mojtaba Hoseiniharouni ^b, Elham Kazemirad ^k, Manzar Bolhassani ^l, Mansour Heidari ^{c,*}

^a Cellular and Molecular Research Center, Basic Health Sciences Institute, Shahrekord University of Medical Sciences, Shahrekord, Iran

^b Department of Molecular Medicine, School of Advanced Technologies in Medicine, Tehran University of Medical Sciences, Tehran, Iran

^c Department of Medical Genetics, Tehran University of Medical Sciences, Tehran, Iran

^d Cancer Biology Research Center, Cancer Institute of Iran, Tehran University of Medical Sciences, Tehran, Iran

^e Cellular and Molecular Research Center, Qom University of Medical Sciences, Qom, Iran

^f Department of Pharmacology and Toxicology, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran

^g Research Center for Molecular and Cellular Imaging, Tehran University of Medical Sciences, Tehran, Iran

^h Department of Pharmacology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran

ⁱ Experimental Medicine Center, Tehran University of Medical Sciences, Tehran, Iran

^j Colorectal Research Center, Iran University of Medical Sciences, Tehran, Iran

^k Department of Medical Parasitology and Mycology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran

^l Department of Medical Genetics, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran

ARTICLE INFO

Article history:

Received 4 August 2017

Accepted 28 May 2018

Keywords:

Colorectal adenocarcinoma

cDNA-AFLP

Nir1

Nir2

Target gene

TGIF2LX

ABSTRACT

Background and study aims: Homeobox-containing genes are composed of a group of regulatory genes encoding transcription factors involved in the control of developmental processes. The homeodomain proteins could activate or repress the expression of downstream target genes. This study was conducted to *in vivo* identify the potential target gene(s) of TGIF2LX in colorectal adenocarcinoma.

Methods: A human colorectal adenocarcinoma cell line, SW48, was transfected with the recombinant pEGFPN1-TGIF2LX. The cells were injected subcutaneously into the flank of the three groups of 6-week-old female athymic C56BL/6 nude mice (n = 6 per group). The transcript profiles in the developed tumours were investigated using the cDNA amplified fragment length polymorphism (cDNA-AFLP) technique.

Results: The real-time RT-PCR and DNA sequencing data for the identified genes indicated that the N-terminal domain-interacting receptor 1 (Nir1) gene was suppressed whereas Nir2 and fragile histidine triad (FHIT) genes were upregulated followed by the overexpression of TGIF2LX gene.

Conclusion: Downregulation of Nir1 and upregulation of Nir2 and FHIT genes due to the overexpression of TGIF2LX suggests that the gene plays an important role as a suppressor in colorectal adenocarcinoma.

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Introduction

Homeobox genes contain a family of regulatory genes encoding transcription factors with a role in the regulation of developmental processes. This group of genes comprises a conserved sequence of 183 nucleotides, the homeobox, which encodes homeodomain, a

60 amino acid helix-turn-helix type of DNA-binding motif [1,2]. Homeodomain proteins are greatly conserved in evolution and are regularly found in animals, plants, and fungi. These proteins have been reported to regulate various embryonic developmental processes such as axis formation, limb development, and organogenesis [3]. A number of gene alterations in homeobox genes have been established to be involved in diseases such as neuroblastoma, leukaemia, and cancer [4–7]. Homeodomain proteins are categorized into diverse classes on the basis of their sequences and conserved domains [5]. The three-amino acid loop extension (TALE) superclass consists of members contains three extra residues between helix 1 and helix 2 of the homeodomain [8]. Some TALE

* Corresponding author at: Department of Medical Genetics, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran. Tel.: +98 21 88953005; fax: +98 21 88953005.

E-mail address: mheidari@sina.tums.ac.ir (M. Heidari).

¹ These authors contributed equally to this work.

homeodomain proteins function as cofactors for the HOX protein clusters are involved in patterning along the anterior-posterior body axis [9,10]. The TALE superclass is classified into four classes in animals: PBC, MEIS, TGIF, and IRO (Iroquois) [4]. TG-interacting factors (TGIFs) comprise a family of TALE-homeodomain proteins that contains several members. Four TGIFs in human are TGIF, TGIF2, TGIF2LX, and TGIF2LY (TGIF2 like on X or Y chromosomes). Previous studies on cultured cells demonstrated that TGIF2 acts as a transcriptional repressor and antagonizes TGF-beta-activated gene expression [11,12]. Moreover, the *tgif2* gene was found to be amplified and over-expressed in various ovarian cancer cell lines [13]. Whether and how it is implicated in the regulation of cell proliferation and its molecular mechanism is not fully known. This study was conducted to identify the possible target gene(s) of TGIF2LX in colorectal adenocarcinoma using cDNA-AFLP, a gene expression analysis assay that is not required prior to gene sequencing.

Materials and methods

Cell culture

Human colorectal adenocarcinoma cell line, SW48, was acquired from the National Cell Bank of Iran (NCBI) affiliated to the Pasteur Institute (Tehran, Iran). The cell line was cultured in RPMI-1640 medium (Gibco, Germany) supplemented with 5% fetal bovine serum (FBS) (Gibco, Germany), 0.03% L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, and 250 ng/ml amphotericin B (Gibco, Germany) at 37 °C in a 5% CO₂ atmosphere, as previously described [14,15].

Cloning of TGIF2LX gene in an expression vector

After amplification of TGIF2LX coding sequence, the PCR product was purified and cloned into pEGFP-N1, a eukaryotic expression vector. The recombinant pEGFPN1-TGIF2LX was transfected into SW48 cells using X-tremeGENE siRNA Transfection Reagent (Roche, Germany). An empty vector pEGFP-N1 was employed as a mock control. Following 48 h, the transient transfection efficiency was evaluated by a UV-fluorescent microscope (Olympus, Japan). The transfected cells were next cultured in the presence of 400 µg/ml G418 (Neomycin antibiotic) (Life Technology, California, USA) for 21 days to select the cells with overexpressing GFP (a marker protein).

Animal study

An animal study was completed as previously explained [16]. Briefly, 6-week-old female athymic C56BL/6 nude mice (n = 6 per group) were obtained from the Omid Institute for Advanced Biomodels (Tehran, Iran). All animal experiments were conducted according to the Ethical Committee of Tehran University of Medical Sciences (ethics code: ERC/S/277). After cell culture, 5×10^6 cells were transfected with the pEGFP-N1 (empty vector) and pEGFP-TGIF2LX. The transfected cells and untransfected cells were injected subcutaneously at a 200 µl volume of the serum-free medium into the flank of the three groups of animals. After three weeks and confirmation of tumour growth, the mice were anesthetized and the tumours were completely isolated.

RNA extraction, Real-time RT-PCR and western blotting

Total RNA was extracted by TriZol isolation reagent (Invitrogen, USA) according to the manufacturer's instructions. Total RNA was DNase-treated with DNase I (Invitrogen, USA) and 5 µg of total

RNA was used to synthesize cDNA fragments using random sequence hexamer primers and Oligo(dT) with a cDNA synthesis system kit (Vivantis, USA), following the manufacturer's protocol. The quality of cDNA was checked using the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) RT-PCR.

Real-time RT-PCR was performed to verify the expression level of TGIF2LX gene in colorectal adenocarcinoma cells. The reaction mixture contained 1 µl of each primer (10 pmol/µl), 1 µl of cDNA and 5 µl of $2 \times$ SYBR Green PCR Master Mix (SYBR Premix Ex Taq II, Takara). The primers are listed in Table 1. The amplification plan included an initial denaturation at 95 °C for 10 min, followed by 45 cycles of a two-stage PCR consisting of 95 °C for 10 s and 60 °C for 30 s. Specificity of primers was verified by observing a single peak in the dissociation curve for each run. All the reactions were done in triplicate.

In order to evaluate the expression level of TGIF2LX protein, Western blot analysis was conducted. Briefly, cell lysates for western blotting were prepared from SW48 cells transfected with the pEGFP-N1 (empty vector), pEGFP-TGIF2LX transfected cells, and untransfected cells. Afterward, SW48 cell lines were subjected to 10% SDS-PAGE electrophoresis and transferred to nitrocellulose membrane (Amersham Biosciences, USA). The membranes were blocked for 1 h with 3% Bovine Serum Albumin (BSA) at 37 °C and incubated with a 1:500 dilution of a polyclonal rabbit anti-TGIFLX antiserum (Santa Cruz Biotechnology, Santa Cruz, USA) for 2 h at room temperature. The membranes were incubated with a goat HRP conjugated anti-rabbit IgG secondary antibody (1:5000) for 1.5 h prior to the development with 4-chloro-1-naphthol (4CN) (Immun-Blot, Bio-Rad Laboratories, USA).

Total RNA extraction and cDNA synthesis

Total RNA of tumours transfected with pEGFPN1-TGIF2LX and empty vector was extracted by the TriZol isolation reagent (Invitrogen, USA) according to the manufacturer's instruction. cDNA was synthesized using 1 µg of total RNA, as described previously [17,18].

cDNA amplified fragment length polymorphism (cDNA-AFLP)

The summary of the cDNA AFLP strategy is shown in Fig. 1. Double strand cDNA (dscDNA) synthesis was carried out using DNA polymerase I enzyme (Thermoscientific, USA) at 16 °C for 2 h. The synthesized dscDNA was purified and quantified by spectrophotometry and gel agarose electrophoresis and subjected to restriction enzyme digestion. About 5 µg of dscDNA was digested with 5 U EcoRI restriction enzyme (Fermentas, Burlington, Canada)

Table 1
Oligonucleotides used in this study.

Oligonucleotides	Name	Sequences
cDNA AFLP Adaptors and Primers	AdL EcoR 1	5'-ACCGACGTCGACTATCCATGAAG-3'
	AdS EcoR 1	5'-AATTCTTCATGG-3'
	Pre-EcoR 1	5'-ACCGACGTCGACTATCCATGAAGAATTC-3'
	S1 EcoR 1	5'-ACCGACGTCGACTATCCATGAAGAATTC-3'
	S2 EcoR 1	5'-ACCGACGTCGACTATCCATGAAGAATTCG-3'
	S3 EcoR 1	5'-ACCGACGTCGACTATCCATGAAGAATTC-3'
Target Genes	S4 EcoR1	5'-ACCGACGTCGACTATCCATGAAGAATTC-3'
	Nir1-F	5'-TATCGGATCGCCAAGTTGCT-3'
	Nir1-R	5'-TTGAGAATCACCCAAAGC-3'
	Nir2-F	5'-GTAGAACTGAACATCGTGGCCG-3'
	Nir2-R	5'-CCACATAGCCGTCTGACAGGA-3'
	FHIT-F	5'-ATCATGGAGGCCATGGAACAC-3'
Housekeeping Gene	FHIT-R	5'-CATCTTCAGCATGTGCGTGG-3'
	GAPDH-F	5'-CACCAGGGTCTGTTTAAAC-3'
	GAPDH-R	5'-ATCTCGCCTCTGGAAGAT-3'

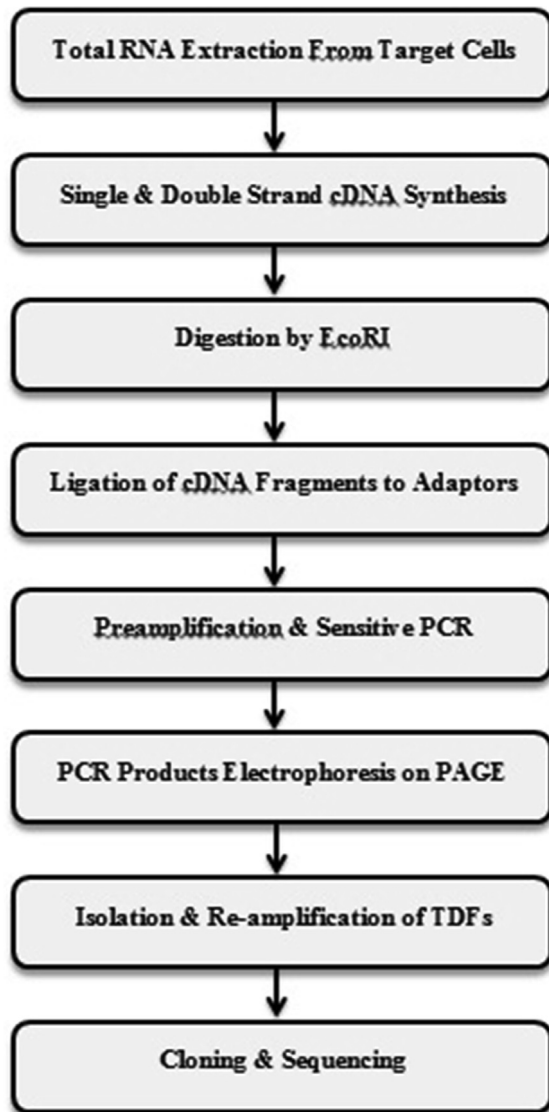


Fig. 1. A summary of steps involved in the cDNA-AFLP method.

at 37 °C for 2 h. EcoRI digested cDNA fragments were ligated to AFLP adaptors (Table 1) using T4 DNA ligase (Vivantis, USA) in T4 DNA ligase buffer in a final volume of 60 µl. Then, the pre-amplification was carried out using pre-EcoRI primers (Table 1) according to the following conditions: the first denaturation at 95 °C for 5 min followed by 30 amplification cycles at 94 °C for

40 s, 60 °C for 40 s, and 72 °C for 1.5 min, and a final extension at 72 °C for 7 min.

The sensitive amplification was conducted as described using sensitive amplification primers (Table 1). Sensitive amplification products were separated on 10% non-denaturing polyacrylamide gel electrophoresis (PAGE) and stained with silver nitrate. Finally, the polyacrylamide gel was evaluated for the presence of expressed transcription-derived fragments (TDFs).

Isolation, cloning, and sequence analysis of TDFs

Differentiated TDFs were separated from the polyacrylamide gel and the eluted DNA was re-amplified by appropriate sensitive primers. The PCR products were checked using 1.5% agarose gel electrophoresis and subsequently cloned into a pTZ57R/T Vector (Thermo scientific, USA). The recombinant plasmids containing unknown DNA were sequenced using M13 forward and reverse primers to determine the identity of the isolated TDFs. Data from sequencing were analyzed in non-redundant nucleic and protein databases Basic Local Alignment Search Tool (BLAST) (<http://www.ncbi.nlm.nih.gov/BLAST>).

Real-time RT-PCR analysis

Real-time RT-PCR was carried out to confirm the differences in expression patterns of TDF-derived genes. Target gene primers were designed by NCBI primer-BLAST program (<http://www.ncbi.nlm.nih.gov/BLAST>) based on the identified genes (Table 1). In order to evaluate the relative gene expression, real-time PCR was performed in triplicate using a CFX96™ Real-Time System (C1000™ Thermal Cycler) (Bio-Rad, Hercules, CA, USA). Reaction mixtures contained 2 µl cDNA, 10 µl of SYBR Green I Taq Mix (Takara, Dalian, China), 1 µl (0.2 mM) of each primer, and 6 µl ddH₂O in a total volume of 20 µl. The amplification condition was as follows: initial denaturation at 95 °C for 3 min, followed by 45 amplification cycles consisting of 95 °C for 5 s, and 60 °C for 20 s. The *GAPDH* gene was used as a housekeeping gene to normalize the data. The relative expression level of the aforementioned gene was analyzed by comparing the cycle thresholds (CTs) of the target genes with *GAPDH* using the $2^{-\Delta\Delta CT}$ method [19].

Results

TGIFLX expression in human colorectal adenocarcinoma (SW48) cell lines

After a 48 h post-infection of SW48 cells with recombinant pEGFPN1-TGIF2LX, pEGFP-N1 (empty vector), and untransfected cells, the cells were examined by UV-fluorescent microscopy (Fig. 2).

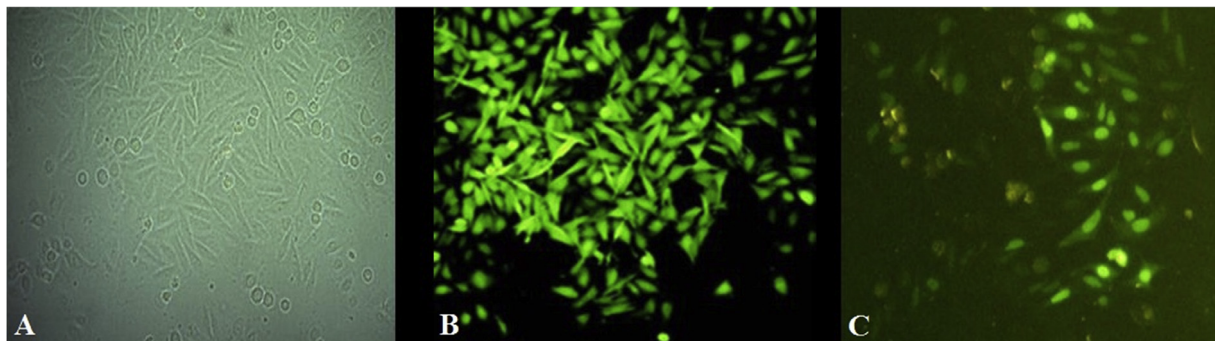


Fig. 2. Fluorescence microscopic analysis of SW48 cell lines; (A): uninfected cells without fluorescence, (B): pEGFP-N1 and C: pEGFPN1-TGIF2LX.



Fig. 3. The mice with the colorectal adenocarcinoma tumour at 21 days of post injection of cells.

Next, 21 days after injection of recombinant pEGFPN1-TGIF2LX, pEGFP-N1 (empty vector), and untransfected cells into the flank of nude mice, tumour tissues were exactly isolated using a stereomicroscope (Fig. 3). There was no significant difference between transfected and non-transfected tumours in visible size.

After confirmation of colorectal adenocarcinoma tumour by histological analysis, transcription and translation of TGIF2LX in tumour cells transfected with pEGFPN1-TGIF2LX were confirmed using quantitative RT-PCR and western blotting, respectively (Fig. 4).

Analysis of the cDNA-AFLP products

The cDNA-AFLP products were separated on 10% non-denaturing PAGE and visualized by silver staining (Fig. 5). Several bands containing TDFs were excised from PAGE, then extracted and re-amplified. Afterward, each desirable band was sub-cloned into TA-cloning vector. After DNA sequencing and analyzing by BLAST, three target genes, including *NIR1*, *NIR2*, and *FHIT* were recognized, which were differentially expressed in the studied samples.

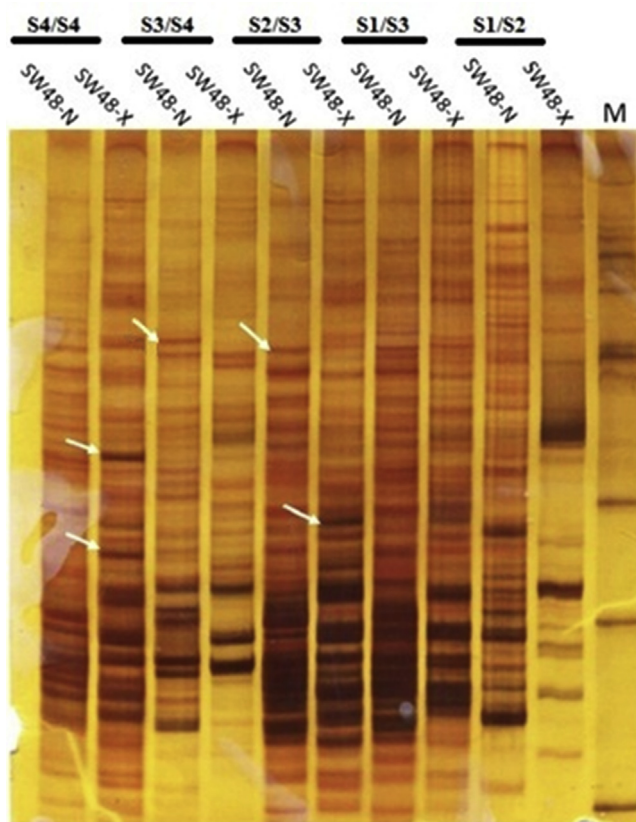


Fig. 5. A representative illustration of cDNA-AFLP on PAGE. Sensitive amplification of cDNA-AFLP on a PAGE from four different primer combinations: S1/S2 EcoRI, S1/S3 EcoRI, S2/S3 EcoRI, S3/S4 EcoRI and S4/S4 EcoRI. The arrows show differentially expressed TDFs. M; (100 bp) molecular size marker.

Validation of cDNA-AFLP results by Real-time PCR

Real-time RT-PCR was carried out to confirm the differential expression of target genes in the tumour cells transfected with the pEGFPN1-TGIF2LX and empty vector. The qRT-PCR results

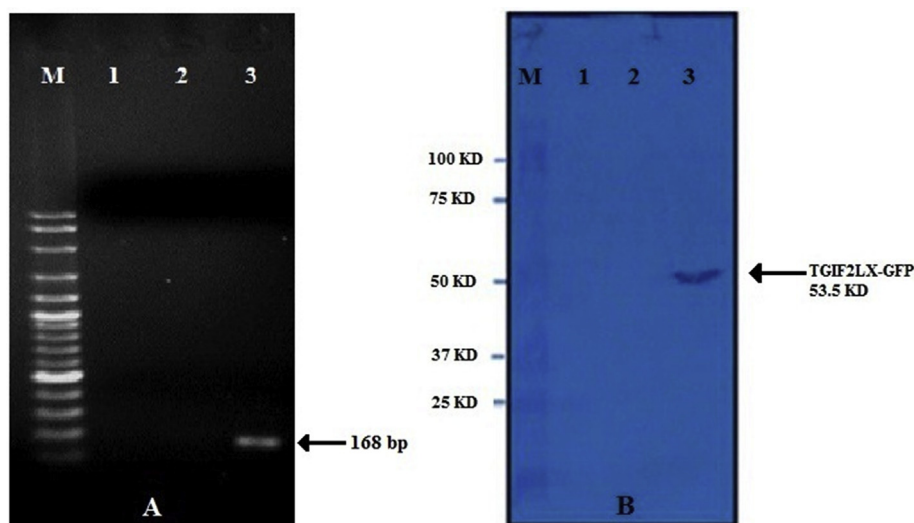


Fig. 4. Expression of TGIF2LX gene in colorectal tumour cells. (A): RT-PCR result with specific TGIF2LX primers. Lane 1: SW48; lane 2: SW48-N; lane 3: SW48-X and lane M: 100 bp size marker (B): Western blotting assay shows significant over expression of TGIF2LX (53.5KDa) in SW48-X cells (lane 3) in comparison with SW48 (lane 1) and SW48-N (lane 2) cells.

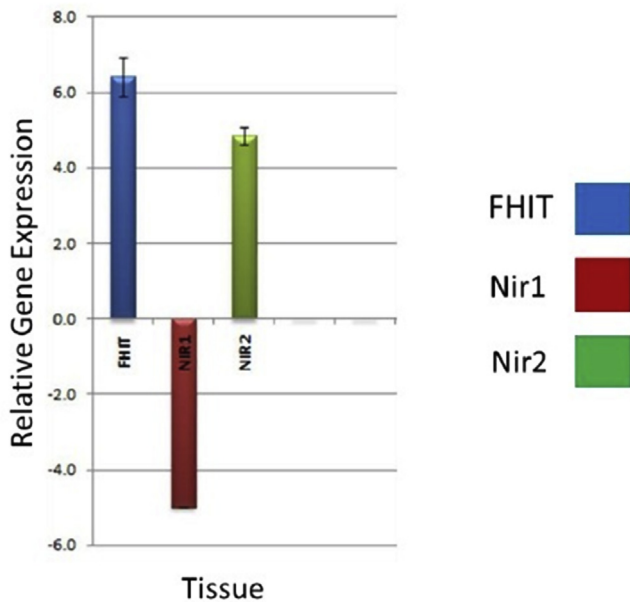


Fig. 6. Relative gene expression pattern of target genes in the cells with and without TGIF2LX by real time RT-PCR. The expression of *GAPDH* was performed to normalize the data. The values are the mean \pm SD of three independent experiments ($P < 0.05$).

indicated significant up-regulation of *FHIT* 6.5-fold and *Nir2* 5-fold and down-regulation of *Nir1* 5-fold in cells transfected with pEGFPN1-TGIF2LX compared to cells with empty vector ($P < 0.05$) (Fig. 6).

Discussion

Colorectal cancer (CRC) is the third most prevalent cancer in men and second most common malignancy in women worldwide [20–22]. CRC is also the third leading cause of cancer-related death, with approximately 600,000 cases annually [23]. Recently, therapeutic strategies for solid tumour treatment have been focused on targeting specified cellular signaling pathways [24,25] that control cell proliferation, apoptosis, and angiogenesis. One of the important signaling pathways in CRC is the transforming growth factor-beta (TGF- β) signaling pathway [26].

TGIF, as a co-repressor for TGF- β -activated Smads, has been revealed to actively repress TGF- β /Smad target genes such as P15, P21, and P27 [27–29]. TGIF plays an important role in holoprosencephaly and ovarian cancer. TGIF2 has been demonstrated to be effective in the progression of ovarian tumours via promoting gene amplification and upregulation of key oncogenic proteins [13,30].

This study was carried out to investigate and identify downstream genes regulated by TGIF2LX. Our hypothesis was that TGIF2LX, like the majority of homeobox genes, could activate or repress downstream target gene(s) directly or indirectly. According to the results, the ectopic expression of TGIF2LX in SW48 cell line resulted in the downregulation and upregulation of *Nir1* and *Nir2* expression, respectively.

N-terminal domain-interacting receptor 1 (*Nir1*), or membrane-associated phosphatidylinositol transfer protein 3, PITPNM3 (also named PYK2), is located on chromosome 17p13.1. This gene contains 20 exons spanning approximately 101 kb of genomic sequence that encodes a protein belonging to phosphatidylinositol transfer protein (PITP) family [30,31]. *Nir1* is a receptor for CCL18 in cellular membrane of cancer cells. The CCL18 specifically binds to *Nir1* to promote migration and metastasis of cancer

cells, especially in breast cancer. In an experimental study, Zhang et al. investigated the correlation of *Nir1* expression with the clinicopathological features of breast cancer patients and the effect of *Nir1* expression on the cell migration and invasion [32]. It was shown that *Nir1* could promote invasion and chemotaxis of cancer cells by binding to CCL18. *Nir1* potentially involves in cofilin recycling and actin polymerization via binding to CCL18. These researchers found that *Nir1* could induce epithelial-mesenchymal transition (EMT) via the PI3K/Akt/GSK3b/Snail signaling pathway through binding to CCL18. Accordingly, *Nir-1* could have a biological role in cancer initiation and progression. Also, this gene potentially involves in cell migration and invasion-related signaling pathways [32]. The human *Nir2* (also known as H-rdgB) is a domain known to contain several retinopathy loci, including Best disease and Bardet-Biedl syndrome I [32].

Moreover, Thian et al. showed that *Nir2* as a novel regulator of the small GTPase Rho plays an important role in actin cytoskeleton reorganization and cell morphogenesis of neuronal cells [32]. It has been revealed that blocking of NIR2 function by microinjection of antibodies against protein NIR2 results in the production of multinucleate cell formation. The *Nir-2*-related genes and corresponding signaling pathways have been revealed to enhance EMT *in vitro* and metastasis in animal models. Also, the upregulation of this gene was shown to contribute to cancer development and progression [32,33].

In the present study, *Nir2* as a TGIF2LX target gene, was upregulated in colorectal adenocarcinoma, grade 4, which resulted in an increase in tissue differentiation and a reduction of oncogenesis. Also, downregulation of *Nir1* and upregulation of *Nir2* caused by TGIF2LX overexpression in SW48 cells suggested that TGIF2LX might suppress tumourigenesis. The results also demonstrated an overexpression of *Fhit* gene in SW48 colorectal adenocarcinoma cells transfected with TGIF2LX, indicating it as a potential target gene. The *Fhit* at human chromosome 3p14.2 is a candidate tumour suppressor gene. Some studies have shown that the reduction of *Fhit* is a triggering factor in carcinogenesis of some malignancies, including lung, esophageal, and liver cancers. The *FHIT* gene belongs to the histidine triad superfamily and encodes a cytoplasmic protein with a hydrolase activity. The fragility of this gene may make it susceptible to rearrangements or carcinogenesis-induced alterations. In other words, the degree of chromosomal fragility may determine the degree of cancer susceptibility [33–37]. Although the detailed mechanism of *Fhit* action is not fully understood, the role of *Fhit* as a tumour suppressive gene has been confirmed in cancerous cells [38]. At the cellular level, *Fhit* has been indicated to promote apoptosis and retard tumour cell proliferation *in vitro* and *in vivo* [39]. In the present study, the upregulation of *Fhit* gene in transfected SW48 indicates the potential role of TGIF2LX in suppression of tumour cells. Based on these data, it can be concluded that TGIF2LX may function as an imperative suppressor colorectal adenocarcinoma cell based on alteration the target genes (*Nir1*, *Nir2*, and *Fhit*), as potential mediators in carcinogenesis. To the best of our knowledge, this is the first report that proposes *Nir1*, *Nir2*, and *Fhit* genes might be regulated by homeodomain protein TGIF2LX in colorectal adenocarcinoma cells. These results may provide a new insight into the multiple cancer signaling pathways underlying colorectal cancer development. It looks promising to use this protein for modulating signals mediated oxidative and degenerative damages. However, further investigations are required to find the precise biological functions of the TGIF2LX gene in cancer cells.

Conflicts of interest

None declared.

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